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Vaccination with soluble Aβ oligomers generates toxicity-neutralizing antibodies

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Abstract

In recent studies of transgenic models of Alzheimer's disease (AD), it has been reported that antibodies to aged beta amyloid peptide 1–42 ($A\beta_{1-42}$) solutions (mixtures of Aβ monomers, oligomers and amyloid fibrils) cause conspicuous reduction of amyloid plaques and neurological improvement. In some cases, however, neurological improvement has been independent of obvious plaque reduction, and it has been suggested that immunization might neutralize soluble, non-fibrillar forms of Aβ. It is now known that Aβ toxicity resides not only in fibrils, but also in soluble protofibrils and oligomers. The current study has investigated the immune response to low doses of $A\beta_{1-42}$ oligomers and the characteristics of the antibodies they induce. Rabbits that were injected with $A\beta_{1-42}$ solutions containing only monomers and oligomers produced antibodies that preferentially bound to assembled forms of Aβ in immunoblots and in physiological solutions. The antibodies have proven useful for assays that can detect inhibitors of oligomer formation, for immunofluorescence localization of cell-attached oligomers to receptor-like puncta, and for immunoblots that show the presence of SDS-stable oligomers in Alzheimer's brain tissue. The antibodies, moreover, were found to neutralize the toxicity of soluble oligomers in cell culture. Results support the hypothesis that immunizations of transgenic mice derive therapeutic benefit from the immuno-neutralization of soluble Aβ-derived toxins. Analogous immuno-neutralization of oligomers in humans may be a key in AD vaccines.

Keywords: ADDLs, Alzheimer's disease, receptors, therapeutic drugs, vaccination.

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Alzheimer's disease (AD) is the most common cause of dementia in older individuals. There is no effective treatment, and the molecular basis for pathogenesis remains uncertain. Multiple factors have been implicated, including CNS inflammation, oxidative damage, and cytoskeletal anomalies (Smith *et al.* 1995; Mandelkow and Mandelkow 1998; Spillantini and Goedert 1998; Finch *et al.* 2001). Increasing evidence, however, favors the hypothesis that a primary cause of AD is neuron dysfunction and death triggered by assembled forms of beta amyloid peptide 1–42 ($A\beta_{1-42}$; Pike *et al.* 1993; Lambert *et al.* 1998; Small 1998; Hartley *et al.* 1999; Golde *et al.* 2000; Klein 2001)

While it has been known for many years that Aβ monomers assemble into large neurotoxic amyloid fibrils

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Abbreviations used: Aβ, beta amyloid peptide; AD, Alzheimer's disease; ADDL, Aβ-derived diffusible ligand; AFM, atomic force microscopy; APP, amyloid precursor protein; β-CD, β-cyclodextrin; BSA, bovine serum albumin; DEAE, diethylaminoethyl; DMSO, dimethyl sulfoxide; ELISA, Enzyme-Linked Immunosorbent Assay; HFIP, hexafluoro-2-propanol; IgG, immunoglobin G; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

(Pike *et al.* 1993; Lorenzo and Yankner 1994), recent studies show that non-fibrillar Aβ-derived toxins also exist. These toxic soluble species comprise *Aβ-derived diffusible ligands* (ADDLs; Lambert *et al.* 1998; Longo *et al.* 2000) and *protofibrils* (Hartley *et al.* 1999; Walsh *et al.* 1999). Protofibrils are relatively large meta-stable structures first described as intermediates en route to full-sized amyloid fibrils (Walsh *et al.* 1997). The ADDLs comprise smaller, soluble $A\beta_{1-42}$ oligomers which form below the critical concentrations needed to form protofibrils and fibrils. Both ADDLs and protofibrils would escape detection in typical neuropathological assays and may account for the imperfect correlation between Alzheimer's progression and insoluble amyloid burden (Terry 1999; Klein *et al.* 2001). These smaller, soluble toxins may also be responsible for the neurological deficits seen in multiple strains of transgenic mice that do not produce amyloid plaques (Mucke *et al.* 2000; Hsia *et al.* 1999; Klein *et al.* 2001).

Inhibiting the toxic impact of assembled $A\beta_{1-42}$ in AD pathogenesis underlies a remarkably promising therapeutic strategy based on immuno-protection. Vaccination of transgenic amyloid-producing mice with solutions of $A\beta_{1-42}$ (prepared under amyloid fibril-promoting conditions) can reduce deposits of fibrillar amyloid and maintain healthy neurite morphology (Schenk *et al.* 1999). Passive vaccination with antibodies induced by Aβ fragments also reduces amyloid burden (Bard *et al.* 2000). The benefits of vaccination also include behavioral improvements (Helmuth 2000; Janus *et al.* 2000; Morgan *et al.* 2000), e.g. age-dependent memory loss in amyloid precursor protein (APP)-transgenic mice is prevented by vaccination, as shown by the radial arm water maze paradigm (Helmuth 2000; Morgan *et al.* 2000). However, in some transgenic mice that carried presenilin 1, as well as APP transgenes, cognitive protection due to vaccination was obtained despite no significant decrease in amyloid burden (Morgan *et al.* 2000). To explain this disconnect, the authors hypothesized that vaccination might target soluble, non-fibrillar toxins derived from Aβ. This explanation for memory protection is consistent with the finding that ADDLs are potent inhibitors of long-term potentiation (Lambert *et al.* 1998; Klein 2001).

Vaccinations of the transgenic mice discussed above were carried out with solutions of $A\beta_{1-42}$ expected to contain varying proportions of fibrils, protofibrils, oligomers and monomers. The current work explores the possibility that oligomers alone might be effective antigens capable of inducing antibodies that neutralize Aβ-derived toxins. Rabbits were injected with solutions that contained only small amounts of oligomers and monomers. The monomers themselves were considered unlikely to be immunogenic because rabbits and humans have the same $A\beta_{1-42}$ sequence (Davidson *et al.* 1992). As predicted, immunized rabbits were found to produce potent antibodies that showed great preference for the assembled, toxic forms of Aβ relative to the physiological monomer. The antibodies have proven useful in assays for ADDL-blocking drugs, in imaging ADDLs bound to cell surfaces, and in detecting ADDLs in brain tissue from humans with AD. The antibodies, moreover, were found to block the toxic action of ADDLs on cultured cells. These results support the hypothesis that therapeutic antibodies could intervene in AD pathogenesis by targeting small soluble toxins, independently of effects on amyloid plaques.

Materials and methods

Materials

Both $A\beta_{1-42}$ and $A\beta_{1-40}$ were obtained from American Peptide (Sunnyvale, CA, USA). Cell culture products were obtained from CellGro (MediaTech, Herndon, VA, USA) and Life Technologies (Grand Island, NY, USA). β-Cyclodextrin (β-CD) containing 12–14% (weight) water (Wacker Silicones Corp., Adrian, MI, USA) was dried at 90°C over P_2O_5 under vacuum (0.5 mmHg) with a dry ice-acetone trap for at least 12 h before use. Unless otherwise indicated, chemicals and reagents were from Sigma-Aldrich (St Louis, MO, USA). The following kits were used: the Roche Boehringer Mannheim Cell Proliferation (MTT) kit (Indianapolis, IN, USA), the Invitrogen Novex Silver Xpress kit (Carlsbad, CA, USA), the Pierce Coomassie Plus and BCA protein assay kits and the Pierce SuperSignal West Femto kit for chemiluminescence (Rockford, IL, USA). The SDS–PAGE gels (16.5% acrylamide, Tris-Tricine) and buffers were from Bio-Rad (Hercules, CA, USA). Antibodies 6E10 (purified form), 6E10Bi and 4G8 were obtained from Senetek (Maryland Heights, MO, USA). 26D6 was a gift of Sibia Corporation (La Jolla, CA, USA). Conjugated secondary antibodies were obtained from Molecular Probes (Eugene, OR, USA) and Amersham (Piscataway, NJ, USA).

Aβ derived diffusible ligand (ADDL) preparation

The Aβ peptides (human sequence) obtained from the manufacturer were greater then 95% pure and were used without further processing. The $A\beta_{1-42}$ was dissolved in hexafluoro-2-propanol (HFIP) and aliquotted to microcentifuge tubes. Hexafluoro-2-propanol was removed by evaporation with traces removed under vacuum; the tubes were stored at −80°C. An aliquot of $A\beta_{1-42}$ was dissolved in anhydrous dimethyl sulfoxide (DMSO) to 5 mM, which was then added to ice-cold F12 medium without phenol red to 100 μM. This solution was incubated at 4°C for 24 h and then centrifuged at 14 000 *g* for 10 min. Centrifugation typically produced a small clear or white pellet, depending on the peptide lot. The supernatant is defined as the ADDL preparation, which comprises fibril-free solutions (by atomic force microscopy) of oligomers (ADDLs) as well as monomers. For experiments using cells, these ADDL preparations were diluted to their final concentration using growth medium without bovine serum. Protein concentration was determined using Coomassie Plus protein assay kit.

MTT Assay

PC12 cells were plated at 30 000 cells/well in 96-well plates and grown overnight. The medium was removed and ADDLs (dose normalized to total Aβ) or vehicle (0.1% or 0.2% DMSO in F12 medium) were added in new medium (F12K, 1% horse serum,

antibiotic/antimycotic). After 4 h at 37°C, the MTT assay was run according to the manufacturer's directions. The MTT (10 μL, 5 mg/mL) was added to each well and allowed to incubate for 4 h at 37°C. Solubilization buffer (100 μL, 10% SDS in 0.01 N HCl) was added and the plate was placed at 37°C overnight. The assay was quantified either at 550–690 nm for Fig. 1 or at 570–690 nm for Fig. 6 on a Dynex MRX plate reader; data were plotted as averages with SEM.

Silver stain

The procedure outlined by the manufacturer (Invitrogen, Carlsbad, CA, USA) was followed. All gels were run using denaturing conditions without reduction. For accuracy at lower molecular weights, Kaleidoscope polypeptide standards (Bio-Rad) were used in Tris-Tricine gels.

Atomic force microscopy (AFM)

Samples were prepared and analyzed using previously published methods (Lambert *et al.* 1998).

Antibody preparation

The polyclonal antibodies were produced and purified by Bethyl Laboratories, Inc. (Montgomery, TX, USA), to whom we sent the initial 24-h supernatant overnight on ice. This material, now equivalent to the '48-h' supernatant (see Results) and still fibril-free by AFM, was diluted 1 : 1 with complete Freund's adjuvant and injected immediately. Booster injections of '48-h' ADDLs in incomplete adjuvant were given every two weeks for a total of 5 boosts. Hyperimmune serum produced in two rabbits was quantified by ELISA against the original antigen solution in a 96-well format. Details of the ELISAs can be obtained from Bethyl Laboratories. Briefly, the immunogen was coated onto microtiter wells by non-specific absorption from a 10-μg/mL solution in sodium carbonate, pH 9, and blocked with 1% bovine serum albumin in Tris buffer (50 mM, pH 8). Immunogen, as shown in results, contains both monomeric and oligomeric peptide, which permits capture of antibodies with a complete range of specificity. Samples were added at various dilutions in 1% bovine serum albumin/Tris and 0.05% Tween 20 + green dye. Anti-rabbit immunoglobin G (IgG) conjugated to horseradish peroxidase was added, and the plate was then developed with 3,3′,5,5′-tetramethylbenzidine (TMB) substrate and H_2O_2. The reaction was stopped with 2 M H_2SO_4 and read at 450 nm. After attainment of an appropriate antibody titer, the animals were bled and the collected antisera were purified using an affinity column. To prepare the affinity column, cyanogen bromide activated agarose, Uniflow 4 (Sterogene Bioseparations Inc., Carlsbad, CA, USA) was loaded with 1 mg of monomer ($A\beta_{1-40}$ in DMSO) on 20 mg gel. Each antiserum batch was passed over the immunosorbent and bound antibodies were eluted with 62 mM citrate buffer, pH 2.3. The flow-through material was further cycled across the column as above until no further antibody could be eluted, as determined by optical density at 280 nm. The eluted material was neutralized, tested for titer in the ELISA, and sent to us. The flow-through material (i.e. material that did not bind to the column) was fractionated using ammonium sulfate precipitation (40%) and diethylaminoethyl (DEAE) fractionation (non-bound from 10 mM phosphate, pH 8, 20 mM NaCl) in batch method to yield an IgG fraction deemed 95% IgG as determined by IEP using anti-rabbit IgG and anti-whole rabbit serum specific antisera (essentially no reactive arcs other than anti-IgG). In a typical processing run (50 mL of antiserum from a single rabbit, two bleeds), approximately 220 mg IgG flowed through the column and about 20 mg of affinity-purified antibodies were eluted from the column. The flow-through material was essentially negative (< 0.3–3.9% of affinity purified material) in the ELISA assay, indicating no loss of alternative Aβ-reactive antibodies. For long-term storage, the samples were maintained Tris-citrate/phosphate, pH 7–8, with 0.1% azide at −20°C in aliquots. The material was diluted to protein concentrations as indicated in each experiment.

Immunoblotting

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to previously published procedures (Zhang *et al.* 1994). In brief, equal concentrations of rat brain homogenate or ADDLs were added to sample buffer and loaded on a 16.5% Tris-Tricine gel. For mixed samples, ADDLs were added to rat brain homogenate just before sample buffer and then placed immediately on the gel. Either Kaleidoscope polypeptide standards (Bio-Rad) or Multimark standards (Invitrogen) were used. The proteins were separated by electrophoresis at 100 V until the dye front reached the bottom of the gel. Proteins were then transferred to nitrocellulose at 100 V for 1 h in the cold. The membrane was blocked for 1 h at room temperature (23°C) with 5% non-fat dry milk in Tris-buffered saline (20 mM, pH 7.6, 137 mM NaCl) with 0.1% Tween 20 (TBST). The sample was incubated with primary antibody in blocking buffer for 1.5 h at room temperature and washed 3 × 15 min. Primary antibody was usually used at a dilution of 1 : 1000 or 1: 2000, equivalent to a protein concentration between 0.3 and 0.6 μg/mL, depending on the antibody used. The membrane was incubated with secondary antibody for 1 h at room temperature (usually a dilution of 1 : 20 000) and washed the same way. Proteins were visualized with chemiluminescence, following manufacturer's directions using only half-strength reagents. Quantification utilized Kodak 1D Image Analysis software for the IS440CF Image Station.

Immunoprecipitation

Protein A Sepharose CL-4B (25 mg/mL, Amersham Pharmacia) was swollen and stored in phosphate buffered saline containing 0.1% SDS, 1% triton and 1% bovine serum albumin (BSA). Before using, the solution was washed twice in the same buffer now including 5 mg/mL deoxycholate and protease inhibitors phenylmethylsulfonyl fluoride (0.175 mg/mL) and aprotinin (100 KIU/mL). The ADDLs (100 pmol) were incubated with or without mouse brain homogenate (100 μg) and with or without antibody (M93, 3.6 μg) for 1 h on ice. Protein A Sepharose (200 μL) was added and the mixture was shaken vigorously at 4°C overnight. The samples were then centrifuged at 500 *g* for 1 min to pellet the beads. Supernatants were then analyzed using immunoblot techniques described above.

Dot blot

Nitrocellulose, marked with pencil to guide sample application, was pre-wetted with Tris buffered saline (see above). Samples of $A\beta_{1-42}$ plus and minus the library of amine derivatives of cyclodextrin (see below) were prepared and incubated at 4°C for various time intervals. After the indicated time, aliquots of each sample (1 μL) were added and the blot was blocked at room temperature for 1 h with 5% non-fat dry milk in TBST. The blot

was then incubated for an hour with primary antibody (M93 at 1 : 1000) in blocking buffer, washed 3 × 15 min and then incubated with secondary antibody (anti-rabbit-IgG-conjugated to horseradish peroxidase, 1 : 10 000 in TBST) for 1 h. The blot was then washed and developed with Pierce SuperSignal chemiluminescence kit, following manufacturer's directions only using half strength reagents.

Per-6-alkylamino-β-cyclodextrin (CD) library preparation

To a solution of per-6-iodo-β-CD (Ashton *et al.* 1996; 40 mg, 0.021 mmol) in dimethylformamide (1 mL) were added imidazole, *N,N*-dimethylethylenediamine and furfurylamine (each 1.47 mmol) and the resulting solution was stirred at 80°C for 24 h. After removal of the dimethylformamide under reduced pressure, the library was precipitated with ethyl acetate, filtered and washed with ethyl acetate. This product was then sonicated in ethyl acetate for 10 minutes to give a fine powder, which was stirred for one hour and then filtered to give the final library preparation after thorough drying *in vacuo*. The library (theoretically containing 2187 compounds) was characterized by electrospray mass spectrometry, showing a doubly charged ion envelope consistent with that calculated for the theoretical distribution.

Preparation of rat hippocampal cultures

Cultures were prepared as described by Brewer (1997). The hippocampus was removed from embryonic day 18 (E18) rat pups and placed in ice-cold Hibernate™/B27 medium. The tissue was cleaned and then dissociated with 6 mL papain (2 mg/mL). Cells were triturated with a fire polished glass pipet, counted and plated on glass coverslips coated with poly-L-lysine (200 μg/mL) and laminin (20 μg/mL). Plating medium was Neurobasal™/B27, supplemented with 0.5 mM glutamine (Sigma) and 1% antibiotic/antimycotic. This procedure usually gave us clean, primarily neuronal cultures that developed long processes.

ADDL Immunofluorescence

Cells were cultured on coated glass coverslips as described above. On the 5th day of culture, ADDLs (0.265 μM, 1 : 200 dilution of 0.24 μg/mL) were added to cells in serum-free medium for 1.5 h at 37°C. Free ADDLs were removed by washing with warm medium. Cells were fixed at room temperature in 1.88% formaldehyde for 5 min, followed by a post-fix for 10 min in 3.7% formaldehyde. Bound ADDLs were identified by incubation with M94 polyclonal antibody (1 : 200) and visualized using anti-rabbit IgG conjugated to Oregon Green−514 (Molecular Probes, 1 : 200). A Nikon Diaphot inverted microscope equipped for epifluorescence was used to view the cells. Images for analysis were captured using MetaMorph imaging software (Universal Imaging Corp., West Chester, PA, USA).

Preparation of human tissue

Samples of frontal cortex, temporal cortex and cerebellum from AD and age-matched control brains were obtained from the Northwestern University Alzheimer's Brain Bank, stored at −80°C and prepared for analysis by a modification of the method of Kawarabayashi *et al.* (2001). Brain tissue (150 mg/mL) was homogenized in 20 mM Tris, pH 7.6, 137 mM NaCl, containing 2% SDS and protease inhibitors (Completegr; mini tablet; Roche, Indianapolis, IN, USA) on ice using a Dounce homogenizer. After centrifugation at 20 000 *g* for 10 min, the supernatant was transferred to another tube and centrifuged at 100 000 *g* for 60 min. The 100K pellet was resuspended in the same extraction buffer. Protein concentration was determined by standard BCA assay. Fractions were stored at −80°C. Results using the 100K pellet suspension are shown in Fig. 5.

Results

Characterization and consistency of antigen

In order to immunize with defined ADDL antigens, we first verified that our preparations consistently provided expected structure and neurotoxicity. Beta amyloid peptide-derived diffusible ligand solutions by definition contain only monomer and toxic oligomers (Lambert *et al.* 1998). To eliminate seeds that promote fibril formation, $A\beta_{1-42}$ from the supplier was first monomerized by dissolving in HFIP and then dried for storage (Stine *et al.* 2000). This monomerized $A\beta_{1-42}$ was used weekly for 8 weeks, reliably giving ADDLs that were at the same concentration (0.24 ± 0.01 mg Aβ/mL). Constituents of each preparation were analyzed by SDS–PAGE and silver staining and shown to consist exclusively of small oligomers and monomers. Figure 1(a) illustrates the composition of one preparation used for immunization. After initial formation of ADDLs (24-h sample), storage at 4°C for another 24 h (48-h sample) caused no change in composition. Atomic force microscopy verified that ADDL solutions were free of protofibrils and fibrils after 48 h, whether stored at 4°C (Fig. 1b) or at 37°C (not shown), confirming previous observations (Lambert *et al.* 1998). Each preparation also was tested for toxicity to PC12 cells as assayed by impact on MTT reduction. The assay is a sensitive marker for ADDL toxicity and monitors disruptions in oxidative metabolism and transport vesicle trafficking (Shearman *et al.* 1994; Liu and Schubert 1997; Liu *et al.* 1998). Whether measured immediately after preparation, or one day later, the ADDL solutions showed consistent potency in blocking MTT reduction (Fig. 1c). Even though toxicity occasionally showed a slight decrease at 48 h, as shown here, differences in toxicity were negligible and maximal at less than 3 μM. Equivalent analyses were carried out for each preparation used for injection. These results established that immunogens were consistent throughout the course of the study with respect to protein concentration, oligomer profile and toxic activity.

ADDL solutions prepared as above (48-h ADDLs, 0.24 mg/mL total protein, see Materials and methods) were mixed with 1 mL complete Freund's adjuvant and injected immediately into two rabbits (0.12 mg protein/animal). Booster injections in incomplete adjuvant were given every two weeks for a total of five boosts. Emergence of hyperimmune serum was detected using an ELISA against the original antigen solution in a 96-well format. The two rabbits were bled three times to obtain antisera which

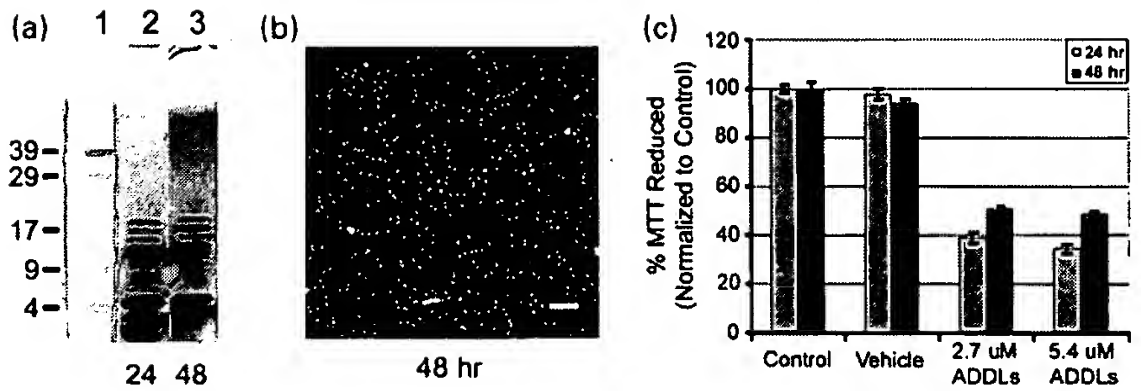

Fig. 1 ADDLs maintain oligomeric profiles and activity with storage at 4°C. (a) Silver stain of initial ADDL preparation and the same preparation 24 h later. $A\beta_{1-42}$ was dissolved in DMSO, then in F12 (see Materials and methods), and incubated at 4°C for 24 h. After centrifugation, the supernatant, which represents the initial ADDL preparation, was transferred to a new tube. The content of each sample was determined at the same time as toxicity was assayed. Supernatant proteins were separated on a Tris tricine gel using SDS–PAGE and visualized with a silver stain. Lane 1: Colored molecular weight markers (not silver stained). Lane 2: Initial ADDL preparation showing abundant monomer, slight dimer, and substantial trimer and tetramer. Lane 3: The same ADDL preparation 24 h later at 4°C showing essentially the same profile. In this image, the uniform gray background of these two lanes is from the colored background of the silver stain. (b) Atomic force microscopy (AFM) of ADDL solution at 48 h. The ADDL preparation at 48 h was applied to clean mica and imaged by AFM (see Materials and methods). Imaged preparations comprise primarily small globular structures, 5–6 nm in size, with a distinct lack of fibrils or protofibrils. The bar represents 400 nm. (c) MTT Assay of initial ADDL preparation and the same preparation 24 h later. The MTT assay was used to assess the biological activity of fresh (24 h) and stored (48 h) ADDL preparations (n equals 4 for each condition with bars equaling standard error of the mean). PC12 cells were incubated for 4 h with ADDLs or vehicle and then assayed for MTT reduction (see Materials and methods). Whether fresh or stored, ADDL preparations caused at least 50% inhibition. Data from above indicate that the 48-h sample, which was used for injection, is similar in structure and toxicity to the initial preparation.

were then purified by affinity chromatography (see Materials and methods).

Specificity of antibodies

The ability of the new antibodies (M93 and M94) to identify various Aβ species was first assessed by immunoblots of synthetic ADDL preparations, which contain abundant monomer as well as smaller amounts of toxic oligomers. Figure 2(a) shows an immunoblot in which identical amounts of ADDLs were probed with decreasing concentrations of M93 antibody. The results exhibit a strong preference for oligomeric species. While M93 can bind monomer, this is evident only at high concentrations of antibody (< 1 : 500 dilution, ~1.2 μg/mL; quantified in Fig. 2b). At 1 : 1000 (0.6 μg/mL), the M93 stains oligomers without staining monomer. In contrast, monoclonal 6E10 at essentially the same concentration (1: 2000 dilution, ~0.5 μg antibody/mL), produces prominent monomer immunoreactivity.

Specificity of the polyclonal antibodies for oligomers in ADDL preparations is in contrast with the non-selective binding of widely used monoclonals 4G8, 26D6 and 6E10. 26D6 (M. Z. Kounnas, personal communication) and 6E10 (Kim *et al.* 1990) recognize similar epitopes of Aβ, aa1–12 and 1–16, respectively; 4G8 recognizes aa17–24 of Aβ (Enya *et al.* 1999). Comparisons showed similar efficacies but marked differences in specificity (Figs 2a and c), with the three monoclonals recognizing monomers as well as oligomeric species. 4G8 also was particularly effective at binding small amounts of dimer. Under equivalent conditions, M94 and M93 recognized only trimer and tetramer (Figs 2a and c). The possibility that binding was non-specific was eliminated by pre-absorbing each antibody with ADDLs for 2 h at 4°C (Fig. 2c, far left lane, M94 shown). The ratio of tetramer/trimer in individual preparations may vary, depending on the peptide lot and on preparation conditions. For example, ADDLs made at very low concentrations of Aβ often show primarily tetramer. An example of this variation is seen in the intensities of trimer versus tetramer in Figs 2 and 3. The polyclonals also were found to bind higher order species of assembled Aβ, including amyloid fibrils (not shown). Specific binding exhibited by the M93 and M94 antibodies thus requires three-dimensional epitopes, determined by the quaternary structure of multimeric Aβ, that are absent from monomeric Aβ.

To determine if the antibodies recognized oligomeric Aβ in its natural state, they were tested for their ability to remove ADDLs from solution by immunoprecipitation. ADDL solutions were treated with antibody, mixed with Protein A Sepharose beads and subjected to low speed centrifugation. This treatment completely eliminated ADDLs from solution (Fig. 2d, second from left lane). Controls lacking antibody did not remove ADDLs (Fig. 2d, left lane).

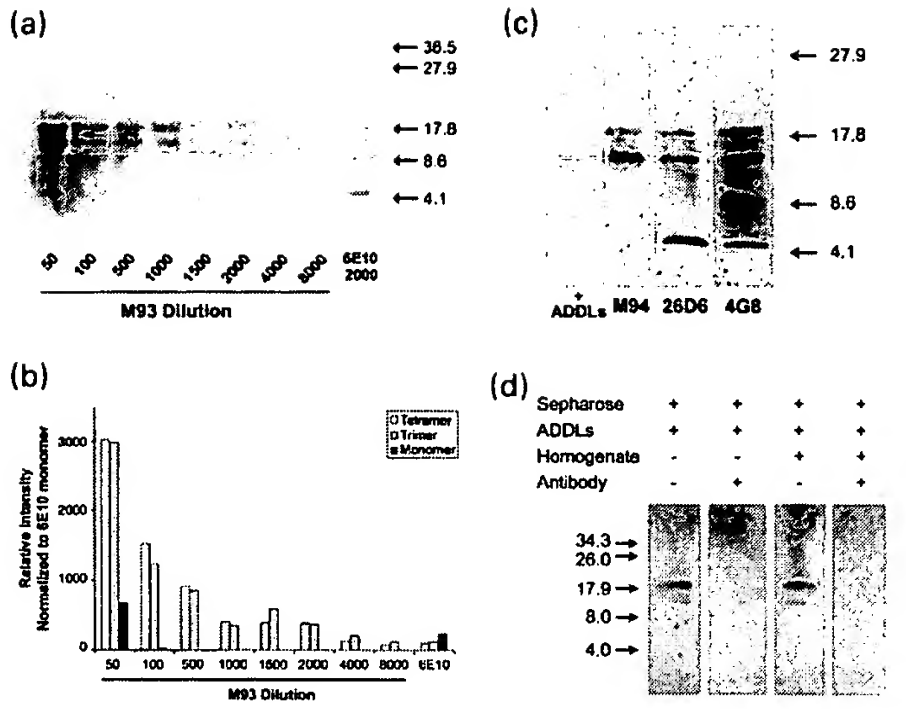

Fig. 2 (a and b) Oligomer-selective M93 antibody detects monomer at hight antibody concentration. (a) Immunoblot. ADDLs (137 pmoles) were separated using SDS–PAGE, transferred to nitrocellulose and probed with decreasing concentrations of M93 antibody (1 : 1000 dilution represents 0.6 μg/mL). Binding was identified with a rabbit secondary antibody conjugated to horseradish peroxidase and visualized using chemiluminescence. A commercial monoclonal antibody, 6E10, unselective for oligomers, is shown for reference (1: 2000 dilution represents 0.5 μg/mL). (b) Quantification of chemiluminescent bands. The intensity of each band was determined by image analysis (Materials and methods) and normalized to the 6E10 monomer band (100%). M93 antibody gave detectable monomer immunoreactivity only at high antibody concentrations (> 6.0 μg/mL). (c) Antibody M94 shows similar preference for oligomers. An ADDL preparation was immunoblotted with antibodies M94, 4G8 and 26D6. Whereas oligomer band intensity was comparable for all three antibodies, 26D6 showed prominent monomer immunoreactivity, while 4G8 also showed monomer and prominent dimer. Antibody concentrations are as follows: 0.5 μg/mL for 6E10 and 4G8; 0.4 μg/mL for 26D6, various concentrations of M93; and 0.9 μg/mL for M94. In the far left lane, M94 was pre-incubated with excess ADDLs (67 μmolar) for 2 h before exposure to the nitrocellulose to assess whether immunoblot binding was non-specific. (d) M93 antibody immunoprecipitates ADDLs from solution, with or without rat brain homogenate. Left two lanes: ADDLs were exposed to M93 or vehicle for 1 h at 4°C, immunoprecipitated with Protein A Sepharose, and the supernatants immunoblotted using M93 (see Materials and methods). Samples with antibody show loss of oligomers. Right two lanes: the procedure above was repeated except the samples also contained 150 μg of normal rat brain homogenate. The second lane from right, without antibody, shows trimer and tetramer, as well as higher molecular weight species. After immunoprecipitation (far right lane), the immunoblot shows no oligomer bands; also no non-specific binding to brain homogenate proteins is evident.

The ability of antibody to remove ADDLs from solution was not blocked by the addition of normal rat brain homogenate (Fig. 2d, right two lanes). We noted that when rat brain homogenate was present, several higher molecular weight bands also were detected in the control preparations. These may be larger oligomers, as previously found in human brain (Guerette *et al.* 2000), or perhaps complexes between ADDLs and second proteins such as ApoE (Munson *et al.* 2000).

In addition, the antibodies have no reaction with homogenate proteins. Lack of non-specific binding of the antibodies to neural proteins was verified using standard immunoblots. Total rat brain homogenate, rat post-mitochondrial membrane fractions and B103 rat CNS neuroblastoma cell homogenate all show no immunoreactive bands when probed with antibody (data not shown).

Assay for assembly blocking drugs

Because M93 and M94 antibodies discriminate assembled forms of Aβ from non-assembled forms (monomer), they could be useful for screening drugs that block assembly of Aβ into ADDLs and higher order species. To test this application, we used a dot blot assay to screen cyclodextrin and libraries of cyclodextrin derivatives. Test compounds were incubated with $A\beta_{1-42}$ (10^{-8} M) on ice for various time periods (Fig. 3). Under control conditions, ADDLs formed rapidly, essentially as fast as could be determined. Cyclodextrin, which previously has been reported to block fibril formation (Camilleri *et al.* 1994), was ineffective in blocking ADDL formation. On the other hand, cyclodextrin derivatives found in a per-6-alkylamino library prevented ADDL formation for at least 4 h. These results were confirmed by analysis of SDS–PAGE immunoblots (Fig. 3,

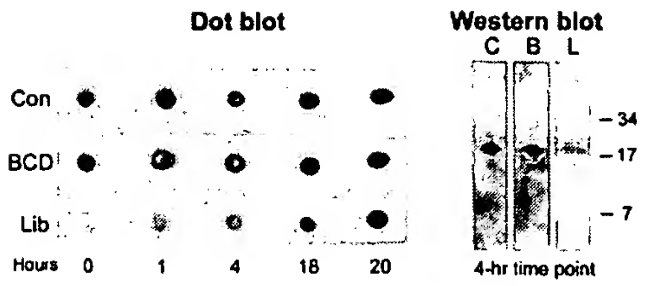

Fig. 3 Delayed formation of oligomers assessed by dot blot assay. Aliquots of a library (Lib) containing amine derivatives of β-cyclodextrin (BCD) were incubated at 4°C with $A\beta_{1-42}$ (10 nM in F12) at a 500 : 1 mole ratio (i.e. 5 μM in parent BCD: 10 nM Aβ). $A\beta_{1-42}$ was also incubated alone (Con) or with the parent BCD compound (5 μM). Left: Dot blot. Samples of these solutions (1 μL) were applied at the indicated times to a previously wetted and marked nitrocellulose membrane. The blot was blocked, exposed to M93 antibody, and developed (see Materials and methods). Right: western blot: aliquots (20 μL) of these same solutions were separated using SDS–PAGE and immunoblotted using M93 antibody (1 : 1000, see Materials and methods). The 4-h time point is shown. Both the dot blot and the immunoblot show decreased amounts of oligomer for at least 4 h in the presence of library.

right) which shows formation of the tetramer, the predominant species, to be reduced by the library. The β-cyclodextrin libraries used in this study contain large numbers of derivatives modified with varied functionality on the cyclodextrin primary face, which may provide a more precise complementary binding interaction with the Aβ peptide and thus reduce Aβ/Aβ interactions. At later times, even the per-6-alkylamino compounds did not prevent ADDL formation.

Immunofluorescence microscopy of oligomer binding to cultured hippocampal cells

We next tested the antibodies for their usefulness in detecting ADDLs bound to cells in culture. This possibility was suggested by their high affinity and minimal non-specific binding in immunoblots. Cultures were prepared from E18 rat hippocampus and incubated with ADDLs for 90 min at 37°C (see Materials and methods). Cells were fixed, incubated with M94 and visualized with a secondary IgG conjugated to Oregon green-514. No signal was seen without ADDLs, but in their presence M94 detected small puncta localized predominantly on neurites (Fig. 4).

Detection of ADDLs in human brain

Results from transgenic mice models and CNS culture paradigms strongly suggest that ADDLs may be involved in AD pathogenesis (Klein *et al.* 2001). This possibility is supported further by initial immunoblots of human brain tissue with the M93/94 antibodies. Brain tissue of Alzheimer's diseased and aged matched control individuals was extracted essentially according to Kawarabayashi *et al.* (2001; see Materials and methods) and assayed for SDS-stable oligomers. Frontal

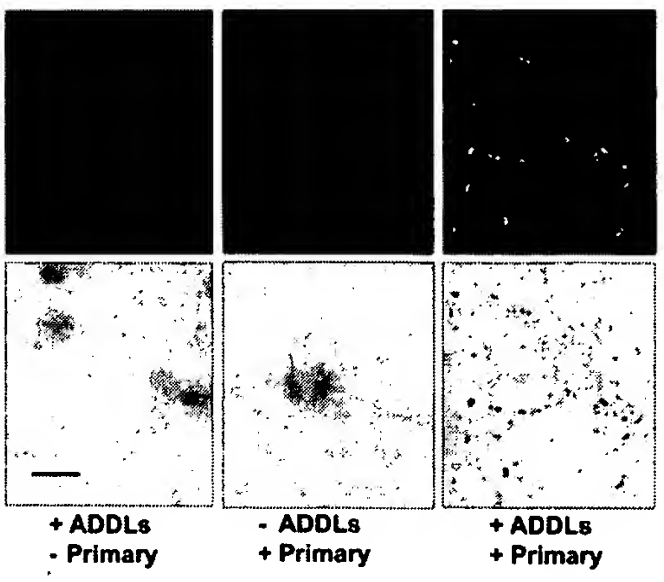

Fig. 4 Immunolocalization of ADDLs bound to cultured rat hippocampal cells. Five-day-old rat hippocampal cultures prepared from E18 embryos were exposed to ADDLs (0.26 μM) for 90 min and fixed for immunofluorescence microscopy (see Materials and methods). Bound ADDLs were identified using M94 antibody and visualized with secondary rabbit IgG conjugated to Oregon green-514. The top panels are immunofluorescence images; the bottom panels are inverted fluorescent images. Left: cultures were treated with ADDLs but no primary antibody. Middle: cultures were treated with vehicle control and M94 antibody. Right: cultures were treated with ADDLs and M94 antibody. No immunoreactivity is found in primary-free or ADDL-free cultures, but bright punctate spots, primarily on neurites, are seen in cultures treated with both ADDLs and M94. The bar in the lower left corner represents 20 microns.

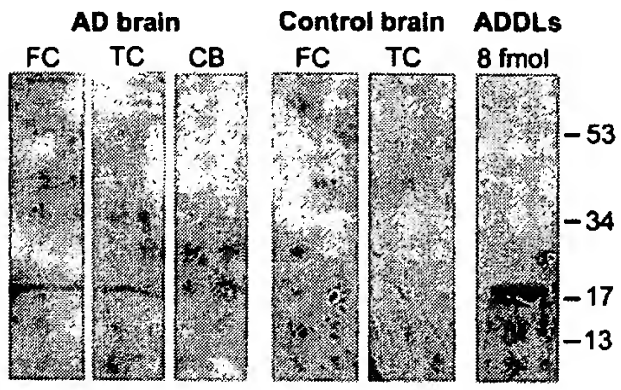

Fig. 5 ADDL immunoreactivity in AD brain extracts. Samples of AD and aged-matched control brains were provided by the Northwestern Alzheimer Brain Bank. Aliquots of tissue (5 μg, see Materials and methods) from the frontal cortex (FC), temporal cortex (TC) and cerebellum (CB) were separated using SDS-PAGE, transferred to nitrocellulose, and immunoblotted using M93. FC and TC of AD brain (left two lanes) show ADDL immunoreactivity (tetramer). Corresponding areas from control brain do not have this band, nor does cerebellum from either AD brain or control brain (not shown). Immunoreactivity that did not enter the gel was also seen in TC and FC of AD brain but not in control brain (not shown). The far right lane indicates the immunoreactivity of 8 fmole of synthetic ADDLs. All lanes were run and developed together on the same immunoblot.

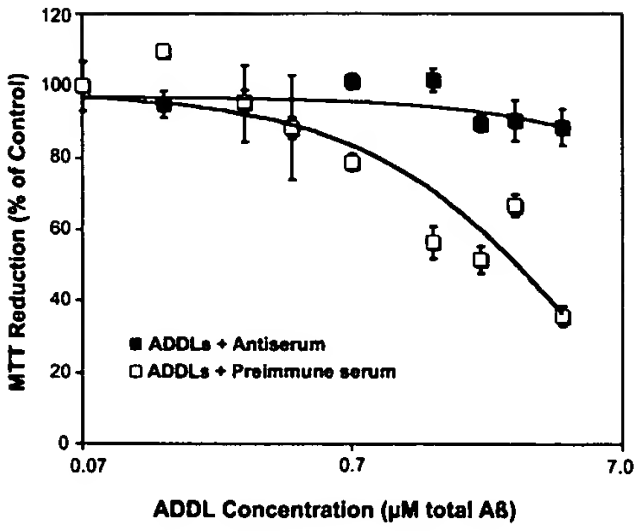

Fig. 6 Toxicity of soluble ADDLs is neutralized by antibody. MTT reduction was assayed in PC12 cells in the presence of increasing concentrations of ADDLs (see Materials and methods). Dose-dependent inhibition by ADDLs was unaffected by incubation with pre-immune serum (□, bottom line). However, pre-incubation with M94 antibody (56 μg/mL = 0.35 μM) inhibits ADDLs toxic activity (■, top line). Antibody is substoichiometric with respect to total Aβ which includes monomer as well as oligomer. Similar results were obtained with clusterin-chaperoned ADDLs (not shown).

and temporal cortex of AD brain showed a distinct band in the Aβ tetramer range, whereas control brain and AD cerebellum did not (Fig. 5). Oligomers thus were present in AD-vulnerable regions but absent from an AD-insensitive region. Quantities of tetramer were small in these extracts, less than 8 fmole/5 μg (compare with synthetic ADDL standard, Fig. 5). The AD frontal cortex of Fig. 5 also shows a faint band at about 110 kDa. Initial results from several tissue samples were variable (not shown). Some exhibited no tetramer, while others showed oligomers at ~55 and/or ~110 kDa. In all AD samples, insoluble amyloid itself was robustly stained at the top of the gel (not imaged). The data confirm that SDS-stable Aβ oligomers can be found in human brain following the onset of AD.

Neuroprotection by antibody

Given the possibility that ADDLs exist in AD brain, we tested whether the antibodies could prevent ADDL neurotoxicity in a cell culture paradigm. Toxicity was assessed by the impact of four hours ADDL exposure on MTT reduction in PC12 cells (Oda *et al.* 1995). Control assays (in the presence of pre-immune serum) showed a dose-dependent ADDL blockade of MTT reduction (Fig. 6, open squares). The curve is right-shifted compared with cell death assays employing hippocampal slices from differentiated brain (Lambert *et al.* 1998), presumably due to differences in assay and cell type. To test for protection, antibodies and ADDLs were incubated together for 2 h before being assayed. The amount of antibody was kept constant (56 μg/mL = 0.35 μM) and the ADDL concentration was increased, resulting in a 1 : 12 molar ratio at the highest ADDL concentration (4.2 μM in total Aβ solute, which comprises monomer as well as oligomers). After pre-incubation with antibody, ADDLs were no longer active (Fig. 6, filled squares). Equivalent results were obtained for a 24-h impact (not shown). In addition, protection occurred whether ADDLs were made with or without clusterin as chaperone (not shown). These results demonstrate a potent ability of ADDL antibodies to neutralize soluble Aβ toxins.

Discussion

Data presented here demonstrate Aβ-derived oligomers (ADDLs) are effective antigens that induce antibodies of analytical and potentially therapeutic value. The antibodies readily recognize assembled forms of Aβ, which are known to be toxic *in vitro*, but they associate poorly with Aβ in its physiological monomeric state. Given their potency and specificity, the antibodies are useful for: (1) screening compounds that delay ADDL formation and (2) imaging the subcellular distribution of ADDLs on cultured hippocampal cells and (3) detecting ADDLs in AD-afflicted brain. In addition, the antibodies neutralize ADDL toxicity in a cell culture paradigm. Because emerging evidence implicates ADDLs in a pathogenic Aβ cascade (Klein *et al.* 2001), the ability to immuno-neutralize soluble toxins may be of significant value for future therapeutic intervention.

A critical property of the new antibodies was their specificity for assembled forms of Aβ, even though the ADDL preparations used to inject rabbits contained a substantial proportion of monomer. Commercial monoclonal antibodies, in comparison, readily bound to the physiological Aβ monomers. Specificity of the ADDL-induced antibodies may be due to the fact that human monomer has the same sequence as rabbit (Davidson *et al.* 1992), while oligomers constituted a novel molecular species. The three dimensional epitopes remain to be elucidated but will be of considerable interest given their relevance to the toxic domains of assembled Aβ. Affinity purification by an $A\beta_{1-40}$ column is consistent with a capacity of the antibodies to bind monomer weakly. At the concentrations used to prepare the column (50 μg/mL, or ~11 μM), $A\beta_{1-40}$ in the absence of seeds would not be expected to make fibrils (Harper and Lansbury *et al.* 1997) or to form stable oligomers (Levine 1995). The inadvertent presence of seeds, however, might have led to multimers assembling on the column. We had anticipated that oligomer-specific antibodies would flow through the $A\beta_{1-40}$ column. The flow-through, however, contained almost no antibodies.

Given their specificity, one practical application of ADDL-induced antibodies would be in screens for compounds that block oligomer formation. A prototype assay in the form of a dot-blot was employed here to screen

cyclodextrin derivatives. The assay is fast, not compromised by the presence of monomer, can readily evaluate many conditions, and uses only small amounts of material. Current results showed that cyclodextrin itself had no effect on $A\beta_{1-42}$ oligomerization, in contrast to earlier reports in which this compound prevented fibril formation in $A\beta_{1-40}$ solutions (Camilleri *et al.* 1994). A per-6-alkylamino-derivatized library, however, was found to delay ADDL formation. The β-cyclodextrin molecule is a cyclic structure with a central hydrophobic pocket. The cyclodextrins used in this study are modified with various functional groups substituted around this structural feature. Certain of these derivatives may provide a precise complementary binding site for interaction with the Aβ peptide, in essence mimicking antibody selection (Solomon *et al.* 1997; Frenkel *et al.* 1999, 2000). Similar to natural antibodies to aged $A\beta_{1-42}$, these particular derivatives could enhance solubility by reducing Aβ/Aβ interactions.

In a second application, the antibodies were used with microscopy to detect ADDLs in association with cells. In hippocampal cell culture experiments, immunofluorescence microscopy showed exogenous ADDLs attached to cells in a highly patterned manner. ADDLs were found in puncta that predominantly localized to neurites. This punctate binding of ADDLs also was seen using the commercially available antibody 6E10 (not shown), which does not discriminate between oligomers and monomers. Existence of the punctate 'hot spots' is consistent with previously proposed receptor involvement in ADDL toxicity (Lambert *et al.* 1998; K. Viola unpublished data).

The third application was to assess the possible presence of ADDLs or ADDL-related molecules in human brain tissue by immunoblots. The specificity and sensitivity of the antibodies make it feasible to carry out this assessment under conditions with high signal-to-noise ratios. Extracts of AD-afflicted brain tissues, but not control brain tissues, showed tetrameric ADDLs. Future experiments will be required to determine if the ADDLs derived from larger unstable structures such as protofibrils. In AD samples probed so far we have noted considerable variability in the size of oligomers found, and it is clear that extensive sampling will be required to determine which species are most abundant and which species correlate best with disease progression. However, dot blot assays indicate clear-cut differences between AD patients and normal controls with respect to the presence or absence of soluble assembled forms of Aβ (L. Chang, unpublished data). Identification of oligomers in AD brain parenchyma is consistent with observations of oligomers in vascular amyloid previously reported (Frackowiak *et al.* 1994). Other groups also have indicated the presence of oligomers in AD brain or in transgenic mouse models (Kuo *et al.* 1996; Roher *et al.* 1996; Enya *et al.* 1999; Lue *et al.* 1999; McLean *et al.* 1999). The current results confirm that SDS-stable Aβ oligomers can be obtained from AD brain. They also establish the important point that these oligomers have three-dimensional epitopes closely similar to those of oligomers prepared *in vitro*, supporting the potential value of vaccination with synthetic ADDLs.

Given the possible presence of ADDLs in AD-afflicted brain, it is especially significant that the M93 and M94 antibodies were neuroprotective. Antibodies that target toxic forms of self-assembled Aβ have gained great interest because of the remarkable recent findings that antibodies against Aβ cross the blood brain barrier and are therapeutic in transgenic mice models of AD (Bard *et al.* 2000; Schenk *et al.* 1999). In some cases, vaccination protocols lead to major loss of amyloid deposits (Schenk *et al.* 1999; Bard *et al.* 2000), and they also can be effective in preventing behavioral decline (Morgan *et al.* 2000; Helmuth 2000; Janus *et al.* 2000). It has yet to be established whether behavioral benefits derive from elimination of amyloid deposits. Morgan *et al.* (2000), for example, reported behavioral protection in animals that show a non-significant decrease in amyloid burden. Current results support the hypothesis that vaccination may provide behavioral benefits by inducing antibodies that neutralize soluble Aβ toxins, previously shown to impair neural plasticity (Lambert *et al.* 1998). These findings, along with the multiple transgenic APP mice models that show behavioral and degenerative losses in the complete absence of amyloid deposits (Mucke *et al.* 2000; reviewed in Klein *et al.* 2001), support the emerging view that AD pathogenesis includes plaque-independent toxicity of oligomeric Aβ. Antibodies that target and neutralize these putative toxins may help to combat memory deficits in early stages of AD. The ability to elicit an immune response with low doses of oligomeric peptides (~50 μg total oligomers/injection) suggests their potential usefulness in therapeutic vaccination protocols.

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